IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of :

Hiroshi YANAGAWA et al. :

Serial No. NEW : **Attn: Application Branch**

Filed January 17, 2002 : **Attorney Docket No. 2001-1921**

MOLECULE ASSIGNING GENOTYPE TO PHENOTYPE AND USE THEREOF
(Rule 1.53(b) Divisional Application of Serial No. 09/284,627, Filed June 2, 1999)

THE COMMISSIONER IS AUTHORIZED TO CHARGE ANY DEFICIENCY IN THE FEES FOR THIS PAPER TO DEPOSIT ACCOUNT NO. 23-0975

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents,
Washington, DC 20231

Sir:

Prior to calculating the filing fee, kindly amend the above-identified U.S. patent application as follows:

In the Specification:

Page 1, below the Title, insert the following:

This application is a Divisional application of U.S. Patent Application Serial No. 09/284,627, filed June 2, 1999, now allowed, which is a 371 of PCT/JP97/03766, filed October 17, 1997, currently pending.

Background of the Invention

line 4, replace the heading with the following new heading:

1. Field of the Invention

Page 4, line 24, replace the heading with the following new heading:

2. Description of the Related Art

Page 9, line 9, replace the heading with the following new heading:

Summary of the Invention

Page 10, replace the paragraph beginning at line 22 with the following paragraph:

Thus, the present invention provides a molecule assigning a genotype to a phenotype, which comprises a nucleic acid portion having a nucleotide sequence reflecting the genotype, and a protein portion comprising a protein involved in exhibition of the phenotype, the nucleic acid portion and the protein portion being directly bound by a chemical bond.

Page 21, line 24, replace the heading with the following new heading:

Description of the Preferred Embodiments

Page 67, line 11, delete the entire heading.

In the Claims:

Kindly cancel claims 1-21 without prejudice.

Please add the following new claims.

22. A library of protein-encoding RNA molecules, said RNA molecules being covalently bonded at their 3' ends to a non-RNA moiety.

23. The library according to claim 22, wherein said non-RNA moiety is DNA.

24. The library according to claim 23, wherein said DNA forms a double-stranded chain of DNA and RNA.

25. The library according to claim 23, wherein part of said DNA forms a double-stranded chain of DNA and DNA.

26. The library according to claim 22, wherein said non-RNA moiety is a double-stranded chain of RNA and short chain DNA.

27. The library according to claim 22, wherein said non-RNA moiety is a combination of DNA and a non-nucleotide moiety.

28. The library according to claim 27, wherein said non-nucleotide moiety comprises one or more polyethylene glycol moieties.

29. The library according to claim 22, wherein said non-RNA moiety is a double-stranded chain of RNA and short chain PNA.

30. The library according to claim 22, wherein said RNA molecules are messenger RNAs.

REMARKS

Favorable consideration is respectfully requested in view of the foregoing amendments and the following remarks.

The specification has been amended to effect changes which were effected in the parent application.

Claims 1-21 have been cancelled without prejudice and replaced with new claim 22-30. The new claims are presented to protect other specific embodiments of the present invention. Support for the new claims is readily apparent from the teachings of the specification and the original claims.

Specifically, support for each of the new claims are as follows.

For claims 22 and 23, the step of synthesizing mRNA from a gene library or a cDNA library to construct an *in vitro* virus genome is described on page 40, lines 10-24 and page 12, line 11 to page 14, line 7, and in particular, page 40, line 13. Further, it is described on page 46, lines 17-23 and page 50, lines 2-3, that *in vitro* virus genome was prepared from a cDNA library and was transcribed to prepare RNA genome. The synthesized mRNA or the prepared *in vitro* virus genome is naturally a library because it is synthesized or prepared from the gene library or the cDNA library.

The step of bonding chimeric spacer composed of DNA and RNA to a 3'-terminal end of RNA obtained by transcription of DNA, the step of bonding a chimeric spacer composed of DNA and polyethylene glycol to a 3'-terminal end of RNA obtained by transcription of DNA, and the step of bonding a spacer composed of double-stranded DNA to a 3'-terminal end of RNA

obtained by transcription of DNA are described on page 12, lines 15-16, page 13, lines 8-10 and page 13, lines 24-26, respectively. Also, the bonding of a non-RNA moiety to a 3'-terminal end of RNA obtained by transcription of DNA is described on page 14, lines 13-20 and page 16, lines 4-12. Each of the bonded products has a spacer comprising a non-RNA substance. In addition, spacers (non-RNA moiety) are described on page 22, line 20 to page 23, line 4. From this description, it is clear that the spacer can be DNA. The bonded product has the non-RNA pause sequence (non-RNA moiety).

For claim 24, the bonding of a spacer composed of DNA and RNA to a 3'-terminal end of RNA is described on page 12, lines 11-16.

For claim 25, it is described on page 37, lines 7-11, that the spacer composed of the double-stranded chain of DNA and DNA is not necessarily double-stranded in its full length.

For claim 26, the preparation of a molecule having a spacer composed of RNA and short chain DNA at a 3'-terminal end of RNA is described on page 14, lines 13-20, page 16, lines 6-13, and page 37, lines 3-23.

For claims 27 and 28, the bonding of a chimeric spacer composed of DNA and polyethylene glycol to a 3'-terminal end of RNA is described on page 13, lines 4-10.

For claim 29, the preparation of a molecule having a spacer composed of RNA and short chain PNA at a 3'-terminal end of RNA is described on page 14, lines 13-20, page 16, lines 6-13, and page 37, lines 3-23.

For claim 30, it is described on page 40, lines 10-17, that the RNA can be messenger RNA.

Applicants have submitted a Sequence Listing in computer readable form as required by 37 C.F.R. 1.821(e). The Sequence Listing contains the identical sequences appearing in the application papers. Thus, the content of the paper and computer readable copies are the same and no new matter has been added.

Attached hereto is a marked-up version of the changes made to the specification by the current amendment. The attached page is captioned "**Version with markings to show changes made.**"

Applicants believe that the present application is now in optimal form for examination. Such action is thus respectfully solicited.

Respectfully submitted,

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Version with Markings to Show Changes Made

MOLECULE ASSIGNING GENOTYPE TO PHENOTYPE AND USE THEREOF

This application is a Divisional application of U.S Patent Application Serial No. 09/284,627, filed June 2, 1999, now allowed, which is a 371 of PCT/JP97/03746, filed October 17, 1997, currently pending.

Background of the Invention

1. ~~Technical~~ Field of the Invention

The present invention relates to a molecule assigning a genotype to a phenotype. More specifically, it relates to a molecule assigning a genotype to a phenotype, comprising a nucleic acid portion having a nucleotide sequence reflecting the genotype and a protein portion comprising a protein involved in exhibition of the phenotype. The molecule assigning the genotype to the phenotype of the present invention is a highly useful substance that can be utilized in evolutionary molecular engineering such as in the modification of enzymes, antibodies, ribozymes and other such functional biopolymers and creation of biopolymers having functions not found in living organisms.

Through advances in biochemistry, molecular biology and biophysics, it has been learned that living organisms are molecular machines which function and propagate by interactions among molecules. Among the characteristics of earth's living organisms, the fundamentals are their preservation of genetic information in DNA nucleotide sequences and their ability to translate this information into functional proteins through the medium of mRNA. Owing to progress in genetic engineering, biopolymers with given sequences, like nucleotides and peptides, can now be easily synthesized. Protein engineering and RNA engineering, today

as utilization of enzymes as industrial catalysts, biochips, biosensors and sugar-chain engineering.

Given the fact that molecular design utilizing structural theory is, as symbolized by the continuing high regard for "screening," still in an unperfected state, the evolutionary technique has a practical value for utilization in selecting useful proteins as a more efficient strategy. Building a "time machine" capable of more efficiently producing evolution in a laboratory, if such were possible, would not only enable modification of enzymes, antibodies (vaccines, monoclonal antibodies etc.) and other existing proteins but also open the way to the creation of enzymes for decomposing environmental contaminants, purifiers and others and new proteins not present in the biological world. If an experimental system for protein evolution can be established, therefore, it can be expected to be aggressively utilizable for application in a wide range of fields including power saving and energy preservation in industrial processes, energy production and environmental preservation. The assigning molecule of the present invention is a highly useful substance in protein modification and other aspects of evolutionary molecular engineering.

2. Description of the Related ~~Background~~ Art

Evolutionary molecular engineering is a field of study that attempts to conduct molecular design of functional polymers by utilizing high-speed molecular evolution in the

molecule that simply binds the genotype and the phenotype in the cell-free systems has a number of advantages including the following: (1) that a huge mutant population approaching that of the ribozyme-type can be synthesized, (2) creation of various proteins without dependence on a host, (3) no problem regarding membrane permeability, and (4) that the 21st code can be used to introduce a non-native amino acid.

Summary of the Invention

An object of the present invention is to provide a molecule comprising a virus-type operation replicon which has the advantages of the aforementioned virus-type strategy molecule, exhibits a higher efficiency than phages, and suffers fewer limitations concerning environmental condition setting, namely, a molecule which should be called "in vitro virus", wherein a nucleic acid and a protein are bound by a chemical bond, that is, a molecule in which a genotype is assigned to a phenotype. More specifically, the present invention has been accomplished in order to provide a molecule exhibiting one-on-one relationship between information and function, which can be utilized for creation of functional proteins and peptides, by performing genotype (nucleic acid) assignment to phenotype (protein) using a cell-free protein synthesis system, and binding the 3'-terminal end of a gene to the C-terminal end of a protein with a covalent bond on ribosome. Further, it is also an object of the present invention to obtain target functional

proteins or peptides through investigation of vast sequence space, which is performed by repetition of selection of molecules that assign genotypes to phenotypes formed as described above (also referred to as "in vitro virus" hereinafter) by the in vitro selection method, and amplification of gene portions of the selected in vitro viruses by the reverse transcription PCR, and further amplification while introducing mutations.

The present inventors earnestly conducted investigations to achieve the aforementioned objects, and as a result, they found that two kinds of molecules that assign a genotype to a phenotype, comprising a nucleic acid and a protein which were chemically bound can be constructed on a ribosome in a cell-free protein synthesis system. They further found that a protein evolution simulation system can be constructed wherein the assigning molecules (in vitro viruses) were selected by the in vitro selection method, gene portions of the selected in vitro viruses were amplified by reverse transcription PCR, and the genes were further amplified while introducing mutations. The present invention has been accomplished based on these findings.

Thus, the present invention provides a molecule assigning a genotype to a phenotype, which comprises a nucleic acid portion having a nucleotide sequence reflecting the genotype, and a protein portion comprising a protein involved in exhibition of the phenotype, the nucleic acid portion and the protein portion being directly bound by a

chemical bond.

According to preferred embodiments of the present invention, there are provided the aforementioned assigning molecule wherein a 3'-terminal end of the nucleic acid portion and a C-terminal end of the protein portion are bound by a covalent bond, and the aforementioned assigning molecule wherein a 3'-terminal end of the nucleic acid portion covalently bound to a C-terminal end of the protein portion is puromycin.

According to another preferred embodiment of the present invention, there is also provided the aforementioned assigning molecule wherein the nucleic acid portion comprises a gene encoding a protein, and the protein portion is a translation product of the gene of the nucleic acid portion. The nucleic acid portion preferably comprises a gene composed of RNA, and a suppressor tRNA bonded to the gene through a spacer. The suppressor tRNA preferably comprises an anticodon corresponding to a termination codon of the gene. Alternatively, the nucleic acid portion may comprise a gene composed of RNA, and a spacer portion composed of DNA and RNA, or DNA and polyethylene glycol. The nucleic acid portion may comprise a gene composed of DNA, and a spacer portion composed of DNA and RNA.

As further aspects of the present invention, there are provided a method for constructing a molecule assigning a genotype to a phenotype, which comprises (a) boding a DNA comprising a sequence corresponding to a suppressor tRNA,

attached at its 3'-terminal end. As for Lanes 2-4, the genomes were translated in a cell-free translation system utilizing rabbit reticulocyte lysate and containing [^{35}S]-methionine at 30°C for 20 minutes. The translation products were analyzed by 11.25% SDS-PAGE.

Figure 11 is a photograph of electrophoresis image showing generation of in vitro viruses in a cell-free translation system. An in vitro virus genome composed of the mRNA encoding the N-terminal half (1-165) of human tau protein, a DNA spacer (105 mer), a peptide acceptor, and [^{32}P]-rCpPur was translated by utilizing rabbit reticulocyte lysate at 30°C for 20 minutes. The translation products were analyzed by 11.25% SDS-PAGE. The bonding of the genome and the protein could be confirmed by digestion with mung bean nuclease. When the translation product (Lane 3) was digested with mung bean nuclease, bands appeared (Lane 4) at the locations corresponding to monomer and dimer (Lane 1) of the N-terminal half of human tau protein (1-165). Lane 2 shows the result for an in vitro virus genome labeled with ^{32}P.

Figure 12 shows process steps of a protein evolution simulation method utilizing in vitro viruses.

Description of the Preferred Embodiments

~~Best Mode for Carrying out the Invention~~

In this specification, some technical terms are used, and those technical terms have the following meanings when herein used. The term "nucleic acid portion" means a bonded

ZZ region of protein A. Therefore, it was confirmed that the in vitro viruses could be selected. Introduction of mutation and amplification can be performed by using the already-established error-prone PCR (Leung, D. W., et al., (1989) J. Methods Cell Mol. Biol., 1, 11-15), Sexual PCR (Stemmer, W. P. C. (1994) Proc. Natl. Acad. Sci. If USA 91, 10747-10751) or the like. Therefore, it was verified that the protein evolution simulation method shown in Figure 12 was feasible.

~~Industrial Applicability~~

According to the present invention, a molecule assigning a genotype (nucleic acid portion) to a phenotype (protein portion), and construction methods therefor are provided. There are also provided a protein evolution simulation method utilizing molecules that assign a genotype to a phenotype (in vitro viruses) constructed according to the present invention, which comprises selecting the in vitro viruses by the in vitro selection method, amplifying the gene portion of an extremely small amount of the selected in vitro viruses by reverse transcription PCR, and further performing amplification while introducing a mutation, and the like. The molecule assigning the genotype to the phenotype, the protein evolution simulation method utilizing it and the like of the present invention are an extremely useful substance or experimental system for evolutionary molecular engineering, i.e., modification of